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Native Chick Laminin-4 Containing the β2 Chain (s-Laminin) Promotes Motor Axon Growth

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Abstract. After denervation of muscle, motor axons reinnervate original synaptic sites. A recombinant fragment of the synapse specific laminin β2 chain (s-laminin) was reported to inhibit motor axon growth. Consequently, a specific sequence (leucine-arginine-glutamate, LRE) of the laminin β2 chain was proposed to act as a stop signal and to mediate specific reinnervation at the neuromuscular junction (Porter, B.E., J. Weis, and J.R. Sanes. 1995. *Neuron.* 14:549–559). We demonstrate here that native chick laminin-4, which contains the β2 chain and is present in the synaptic basement membrane, does not inhibit but rather promotes motor axon growth. In native heterotrimeric laminin, the LRE sequence of the β2 chain is found in a triple coiled-coil region that is formed by all three subunits. We show here that the effect of LRE depends on the structural context. Whereas a recombinant randomly coiled LRE peptide indeed inhibited outgrowth by chick motoneurons, a small recombinant triple coiled-coil protein containing this sequence did not.

REGENERATING motor axons preferentially reinnervate original synaptic sites that cover only ~0.1% of the muscle fiber surface (Letinsky et al., 1976). Factors responsible for this selectivity are likely to be components of the synaptic basement membrane, because, if the muscle fibers have been killed as well, motor axons still grow back to former synaptic sites on basement membrane "ghosts" (Sanes et al., 1978). The laminin β2 chain (s-laminin) which is enriched at the synaptic basement membrane of the neuromuscular junction was claimed to be involved in this targeting (Hunter et al., 1989*b*; for nomenclature see Burgeson et al., 1994). Laminins are glycoproteins of ~600-900 kD molecular mass which are abundant in basement membranes (Timpl and Brown, 1994). Each laminin molecule is composed of one α, one β, and one γ chain which have been classified according to their sequence homology and domain organization (Engel et al., 1994). Several members encoded by different genes have been cloned for any of these three types of subunits (see Ryan et al., 1994; Timpl and Brown, 1994; Iivanainen et al., 1995; Miner et al., 1995), and at least seven heterotrimeric laminin isoforms have been isolated so far (Timpl and Brown, 1994). The different subunits, and consequently, the resulting laminin isoforms, have distinct tissue distributions and their expression is developmentally regulated (e.g., Ekblom et al., 1990; Engvall et al., 1990; Sanes et al., 1990; Noakes et al., 1995*b*). For example, laminin with α2 and γ1 chains are found all over the muscle basement membrane surface whereas those with β1 and β2 chains are distributed in a mutually exclusive manner. The β1 subunit is found only extrasynaptically whereas the β2 chain is specific for the basement membrane of the synaptic cleft and of the myotendinous junction (Engvall et al., 1990; Sanes et al., 1990). The β2 chain has originally been discovered by the use of antibodies that specifically recognize synaptic basement membranes (Hunter et al., 1989*b*). Subsequently, it was shown that a laminin β2 chain–derived peptide, Leu-Arg-Glu (LRE)[1], is selectively adhesive for motoneurons while inhibiting their neurite growth (Hunter et al., 1989*a*; Porter et al., 1995). Moreover, recombinant laminin β2 chain was not adhesive for sensory neurons or PC12 cells, nor did it inhibit their neurite outgrowth (Hunter et al., 1989*a*; Porter et al., 1995). These studies led to the hypothesis that the β2 chain could act as stop signal specific for growing motor axons, and that this might at least in part explain the preferential reinnervation of original muscle endplates. The subsequent differentiation into a nerve terminal might be a consequence of growth cone arrest, or it might also be mediated by LRE (Porter et al., 1995). Laminin β2 chain deficient mice show aberrant differentiation of motor nerve terminals

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1. *Abbreviations used in this paper*: BDNF, brain derived neurotrophic factor; CD, circular dichroism; CG, ciliary ganglion; CMP, cartilage matrix protein; LRE, Leu-Arg-Glu.

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(Noakes et al., 1995*a*), implying that this subunit is indeed involved in their differentiation.

However, the in vitro data concerning the adhesivity and inhibitory action of the laminin β2 chain have been generated by the use of recombinant fragments and synthetic peptides of a single laminin chain. The LRE sequence lies within the triple-stranded coiled-coil region of the assembled, trimeric laminin molecule. It is therefore important to test the activity of native laminin containing the β2 chain. The laminin isoform present at the neuromuscular junction is likely to be laminin-4, composed of the α2, the γ1, and the β2 chain (Sanes et al., 1990). We report here that native chick laminin-4 does not inhibit but rather promotes neurite outgrowth by motoneurons. Moreover, this study investigates the influence of the structural context of the LRE sequence on its inhibitory activity.

Materials and Methods

Antibodies

Monoclonal antibody (mAb) 11B7 directed against the chick laminin γ1 chain, and mAb 8D3 recognizing the chicken α2 chain were described elsewhere (Brandenberger and Chiquet, 1995). Monoclonal antibody C4 against the rat laminin β2 chain (Hunter et al., 1989*b*) and mAb JG22 (Greve and Gottlieb, 1982) against the chicken integrin β1 subunit were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences (Johns Hopkins University, Baltimore, MD) and the Department of Biological Sciences, University of Iowa (Iowa City, IA), under contract N01-HD-2-3144 from the National Institute of Child Health and Human Development.

The following anti-chick integrin antibodies were kind gifts: polyclonal rabbit anti-α1 integrin antiserum (Syfrig et al., 1991) by Dr. Mats Paulsson (University of Köln, Köln, Germany), polyclonal anti-α3Ex IgG (Weaver et al., 1995), and polyclonal anti-α8Ex IgG (Müller et al., 1995) against the extracellular part of the integrin α3 and α8 subunit, respectively, by Dr. Louis F. Reichardt (University of California, San Francisco, CA), and polyclonal anti-α6Ex IgG (deCurtis and Reichardt, 1993) by Dr. Ivan deCurtis (San Raffaele Hospital, Milano, Italy).

Laminin Isoforms

Mouse laminin-1 (EHS-laminin) was purified from mouse Engelbreth-Holm-Swarm sarcomas as described (Timpl et al., 1979). Chick laminin was purified from adult chick heart by wheat germ agglutinin affinity chromatography as published (Brandenberger and Chiquet, 1995). From this preparation, isoforms laminin-2 and laminin-4 were separated by sequential immunoaffinity chromatography using subunit-specific monoclonal antibodies as described (Brubacher et al., 1991; Brandenberger and Chiquet, 1995).

Laminin concentration in stock samples was determined by UV adsorption at 280 nm using the extinction coefficient measured for mouse laminin-1, $\epsilon = 0.746$ ml mg^{-1} cm^{-1} (Paulsson, 1988). Concentrations of laminin isoform dilutions were checked by a sandwich ELISA. Microtiter wells were coated overnight at 4°C with 20 μg/ml mAb 11B7 (capture antibody) in 50 mM Na_2CO_3, pH 9.6. Wells were blocked with 1.5% BSA in 150 mM NaCl, 20 mM Tris-HCl, pH 7.4 (TBS) for 1 h at room temperature. The following incubations were done in blocking buffer. After blocking, wells were incubated with serial dilutions of laminin isoforms, followed by rabbit anti-chick laminin antiserum 245 (1:200; Brubacher et al., 1991) and peroxidase-labeled goat anti–rabbit IgG (1:3,000; Biorad, Glattbrugg, Switzerland). Wells were developed with 0.4 mg/ml 1,2-phenylenediamine, 0.03% H_2O_2 in 100 mM citric acid, 200 mM Na_2HPO_4, pH 5.0, for 10 min. For dilution series starting at the same concentration of chick laminin-2 and -4, respectively, the OD_{488} curves overlapped (not shown).

Neuronal Cell Culture

Chick ciliary ganglion (CG) neurons were isolated from embryonic day 8–9 (E8-9)-old white leghorn chick embryos and dissociated in 0.1% trypsin (Gibco, Paisley, Scotland) for 15 min at 37°C. After trituration, cells were cultured in minimal essential medium (Gibco) supplemented with 10% FCS (Gibco), 3% E17 eye extract, and 50 μg/ml gentamycin (Gibco). About 1/6 of the cells from one ganglion were plated per well in a 96-well dish. Cells were incubated at 37°C in 7.5% CO_2 for 8 h.

Chick spinal cord motoneurons were prepared as described (Dohrmann et al., 1986). Briefly, spinal cords were isolated from E5 chick embryos, trypsinized in 0.1% trypsin in Dulbecco's PBS plus 1% glucose for 30 min at 37°C and then triturated. The resulting single cell suspension was centrifuged through a 3-ml cushion of 3.5% BSA (Serva, Heidelberg, Germany) in L-15 medium (Gibco), pH 7, for 15 min at 100 g. The cell pellet was resuspended in PBS containing 0.5 mM EDTA. The cell suspension was layered on top of 3 ml of 6.8% metrizamide (Sigma, Buchs, Switzerland) in L-15, pH 7, and centrifuged for 20 min at 520 g. The cells at the interface were collected in a small volume, diluted in medium, and recentrifuged. They were resuspended in DMEM (Gibco) supplemented with 2.5% chicken embryo extract, 10% FCS, and 50 μg/ml gentamycin (complex medium) and plated. Alternatively, spinal cord motoneurons were cultured in defined medium consisting of Ham's F-12 (Gibco) with 1 mg/ml BSA and 10 ng/ml recombinant human brain derived neurotrophic factor (BDNF; Calbiochem, San Diego, CA) similar to Henderson et al. (1993). In both complex and defined medium, spinal cord motoneurons were incubated at 37°C in 7.5% CO_2 for 24–48 h.

Preparation of Culture Substrata

96-well plates were coated with laminin isoforms at indicated concentrations for 2 h at 37°C. Wells were blocked with 5 mg/ml BSA in PBS (PBS/BSA) for 30 min at 37°C and washed with PBS. The relative amounts of laminin-2 and -4, respectively, bound to the culture substratum, were determined by a solid phase radioimmunoassay. To culture wells coated with laminin and blocked with BSA as described above, mAb 11B7 (20 μg/ml in PBS/BSA) was added for 1 h at 20°C. This antibody binds to the γ1-chain of both laminin isoforms with the same avidity (see above; sandwich ELISA assay). After washing with PBS/BSA, wells were incubated with ^{125}I-labeled goat anti–mouse IgG (10 nM in PBS/BSA; 1 h at 20°C). They were washed again and counted in a γ-counter. For example, in one such experiment the following cpm values were found for laminin-2 and laminin-4, respectively, coated at different concentrations: 4644 and 4080 (3 μg/ml), 2168 and 2105 (1.5 μg/ml), 1046 and 1247 (0.8 μg/ml), 604 and 704 (0.4 μg/ml), and 353 and 422 (0.2 μg/ml). Background values (3 μg/ml laminin-2 or -4; no first antibody) were 256 and 250 cpm, respectively. We have determined earlier that ~60% of the applied laminin binds to the culture plastic within the used concentration range, independently of the isoform (Brandenberger and Chiquet, 1995).

Chick CG neurons were plated on tissue culture plastic directly coated with laminin isoforms and then blocked with PBS/BSA as described above. Chick spinal cord motoneurons were cultured on round 12-mm glass coverslips which were pretreated with 1 mg/ml poly-L-lysine (Sigma, M_r = 15,000) for 15 min, washed well with PBS, and then coated with a 50-μl drop of laminin isoforms at indicated concentrations for 1 h at room temperature. After washing with PBS, the coverslips were blocked with PBS/BSA for 30 min. To prepare a patterned substratum (Goodman and Newgreen, 1985), glass coverslips were coated with one laminin isoform and incubated for 1 h at room temperature. The coverslips were washed with PBS and blocked with PBS/BSA for 30 min. Stripes were scraped free of protein with a plastic pipette tip in perpendicular directions to create a rectangular pattern. The second laminin isoform was coated for 1 h and the coverslips were blocked again with PBS/BSA for 30 min. After washing with PBS, the coverslips were placed into wells of a 24-well plate containing culture medium, and cells were plated.

For neurite outgrowth inhibition experiments, wells of 96-well plates were coated with methanol-solubilized nitrocellulose as described (Lagenaur and Lemmon, 1987; Porter et al., 1995). Fusion protein was mixed with laminin-1 to give a final concentration of 20 μg/ml laminin and the desired concentration of fusion protein. The solution was coated for 2 h at 37°C. Wells were blocked and washed as described above before cells were added.

Analysis of Neurite Outgrowth

CG neurons were analyzed after 8 h. Spinal cord motoneurons were evaluated after 24 and 48 h since they showed a considerable lag phase before sprouting. For analysis, cells were fixed with 3.5% formaldehyde (Merck, Darmstadt, Germany). 1–3 randomly chosen fields per well were photographed and at least three wells per substrate were analyzed. Cells with

processes longer than one cell diameter were considered as neurons bearing neurites.

Neurite lengths were determined from projected negatives using a graphics tablet and analyzed similarly to Chang et al. (1987). Neurites were only measured if they had not contacted other neurites. Data are plotted as the percentage of neurons (vertical axis) with neurites longer than a given length (horizontal axis). The nonparametric Wilcoxon test was used to determine whether neurite length or percentage of sprouting neurons were statistically different between samples. $P < 0.05$ was considered to be significant.

Immunofluorescence

For immunofluorescence, mAb C4 was added to the cultures to give a final concentration of 20 μg/ml. After 1 h at 37°C, cells were washed with PBS/BSA, and fixed with 3.5% formaldehyde. Cell cultures were washed with PBS, blocked with PBS/BSA for 30 min, and incubated with TRITC-labeled goat anti-mouse IgG (Cappel, Durham, NC) diluted 1:100 in PBS/BSA for 1 h. Cells were mounted in glycerol containing 10 mg/ml *n*-propylgallate (Sigma) and 20 mM sodium phosphate, pH 7.4.

Fusion Proteins

Fusion proteins containing, at their COOH termini, a rat laminin β2 chain–derived decapeptide, LREQVGDQYQ, and a mutated peptide, QREQVGDQYQ, were generated as follows. Two partially complementary oligonucleotides, 5′-CCCGGATCCCTG(or CAG)CGTGAACAGGTTGGTGACCAGTACC-3′ and 5′-CCCGAATTCTTATTACTGGTACTGGTCACCAACCTGT-3′, were synthesized based on the codon usage for enteric bacterial highly expressed genes (Gribskov et al., 1984). The oligonucleotides were designed to contain a BamHI restriction site at the 5′ end and a translation stop signal TAA followed by a EcoRI site at the 3′ end. The annealed oligos were extended with *AmpliTaq* DNA polymerase (Roche Molecular Systems, Alameda, CA) to give the DNA fragments encoding for the peptides. The fragments were ligated into the expression vector pPEP-T at BamHI–EcoRI sites. This special vector can be obtained from the authors. It encodes a histidine-tagged 6-kD carrier protein providing a thrombin cleavage site. Colonies of transformed *E. coli* JM109(DE3) bacteria (Promega, Madison, WI) were tested for the production of recombinant protein. Fusion proteins of the expected size of ~7 kD were purified to homogeneity by affinity chromatography on Ni^{2+}-Sepharose (Novagen, Madison, WI) under denaturing conditions as described (Hoffmann and Roeder, 1991). Recombinant plasmid DNA was verified by Sanger dideoxy sequencing.

The carboxy-terminal domain of chicken cartilage matrix protein (CMP) was generated from two synthetic, partially complementary oligonucleotides, 5′ - GAAGAAGATCCGTGCGAATGCAAAGCATCGTGAAATTCCAGACAAAGTGGAAGAACTGATCAACACCCTGCAG-3′ and 5′-GATGATTTTGTTTTTCCAGCGCTTCGATACGTTTCGCCACCGCTTCCAGTTTCTGCTGCAGGGTGTTGATCAGTTC, which were synthesized according to the *E. coli* preference codon usage. Extension of the annealed oligonucleotide with *AmpliTaq* DNA polymerase produced a DNA fragment coding for residues 451-492 of chicken CMP. Additional oligonucleotides were designed to introduce by PCR a NdeI site at the 5′ end (5′-CCTCCCATATGGAAGAAGATCCGTGCGAATG-3′) and a BamHI site and the stop codon TAG at the 3′ end (5′-CCCGGATCCTAGATGATTTTGTTTTCCAGCGC-3′). The amplified product was ligated into the pET-15b expression vector (Novagen) at NdeI–BamHI sites. To obtain a COOH-terminal CMP domain that contains an LRE sequence, the amino acids 479 (aspartate) and 480 (alanine) were mutated to Arg and Glu, respectively, using the site-directed mutagenesis method by Ho et al. (1989). Two fragments, having overlapping ends, were generated from the recombinant CMP-pET-15b plasmid by PCR using two sets of primers (T7: 5′TAATACGACTCAACTATAGGG, 5′-CGCCACTTCACGCAGTTTCTGCTGCAGGGTGT-3′, and T7t: 5′-GCTAGTTATTGCTCAGCGG-3′, 5′-GCAGCAGAAACTGCGTGAAGTGGCGAAACGTATCGAAGC-3′). After denaturation and reannealing, strands from the two products acted as primers on each other. Extension followed by PCR amplification using the primers described above to generate NdeI and BamHI sites resulted in the final product coding for the CMP assembly domain containing a LRE sequence at position 478-480 (rCMP-LRE). This product was ligated into the pET-15b vector. Recombinant plasmid DNA was verified by Sanger dideoxy sequencing.

Recombinant protein was expressed in *E. coli* strain JM109(DE3) and purified as above.

Protease Cleavage of Recombinant Protein

Affinity-purified proteins were dialyzed against thrombin cleavage buffer (20 mM Tris-HCl, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$). Proteolytic cleavage was carried out for 2 h at room temperature using human thrombin (Sigma) at a concentration of 5 U/mg recombinant protein. Peptides LRE and QRE and the CMP assembly domain rCMP and rCMP-LRE were separated from the carrier protein or the histidine tag, respectively, by repeating the affinity chromatography step.

SDS-PAGE

Standard SDS-PAGE was done according to Lämmli (1970). Tricine-SDS-PAGE was performed according to Schägger and von Jagow (1987) on 12 cm × 13 cm slab gels. Gels were stained with Coomassie Brilliant blue (BioRad Labs, Richmond, VA).

Circular Dichroism Measurements

Circular dichroism (CD) spectra were recorded on a Cary 61 spectropolarimeter equipped with a thermostatted quartz cell (Hellma, Muelheim, Germany) of 0.1 cm pathlength. Samples were prepared in 5 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl. Circular dichroism was recorded with an analogue digital converter (National Instruments, Austin, TX). Data reduction analysis was made with the programs LABView (National Instruments) and Sigma Plot (Jandel Scientific, Corte Madera).

Analytical Ultracentrifugation

Analytical ultracentrifugation was carried out as described (Kammerer et al., 1995).

Protein Sequence Analysis

Coiled-coil propensities of the rat laminin β2 chain were scored with the COILS2 algorithm, a modified version of the COILS program (Lupas et al., 1991). A scanning window size of 21 residues and the MTIDK sequence profile were used. In addition, residues at positions "a" and "d" were weighted 2.5 times higher than other residues to place increased emphasis on the continuity of the hydrophbic repeat pattern.

Results

Characterization of Chick Laminin-2 and Laminin-4

Laminin isoforms used for this study are shown on nonreducing SDS-PAGE in Fig. 1. They were isolated from chick heart by sequential immunoaffinity chromatography to mAbs specific for individual chick laminin subunits (Brandenberger and Chiquet, 1995). A laminin isoform mixture was obtained from Sepharose-coupled mAb 11B7 directed against the common γ1 chain. Laminin isoforms with an α2 subunit, i.e., laminin-2 (α2β1γ1) and laminin-4 (α2β2γ1), were retained on a mAb 8D3 affinity column. These two isoforms were separated over a column coupled with mAb C4 (Hunter et al., 1989*b*) which recognizes the rat and chick β2 chain (s-laminin). Bound laminin-4 reacted on immunoblots with mAbs to laminin α2, β2, and γ1 subunits (Brandenberger and Chiquet, 1995). Laminin-2 in the flowthrough was reapplied twice to the mAb C4 column to deplete it of laminin-4. Consequently, it was still recognized by mAbs 11B7 and 8D3 but no longer by mAb C4 (Brandenberger and Chiquet, 1995).

On silver-stained SDS-PAGE under nonreducing conditions, chick laminin-2 appeared as a single homogeneous band of ~850 kD (Fig. 1, LN2). In contrast, the chick laminin-4 preparations reproducibly exhibited two bands (Fig. 1, LN4). The upper band is the full size protein which migrates slightly faster than laminin-2, in accordance with the

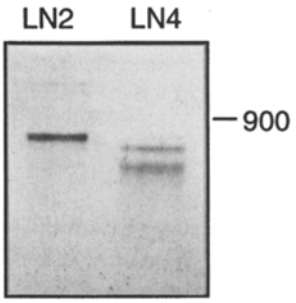

Figure 1. Chick laminin-2 (*LN2*) and laminin-4 (*LN4*) preparations used for neurite outgrowth experiments. Silver-stained 3–15% acrylamide gradient SDS-PAGE run under nonreducing conditions. Adjacent lanes were loaded with equal amounts of protein (as determined by UV absorption). The position of nonreduced mouse laminin-1 (900 kD) is indicated. For details of purification, see text.

smaller size of the β2 as compared to the β1 subunit (Hunter et al., 1989*a*). The lower band was shown immunologically to be laminin-4 as well. The smaller size of this species is due to a truncation of its α2 subunit; however, it contains a full size 190-kD β2 chain (Brandenberger and Chiquet, 1995).

Bands corresponding to the other respective laminin isoform were barely if at all detectable in our two preparations (Fig. 1). Samples as shown in Fig. 1 were employed in the functional assays described in the next paragraphs.

Native Chick Laminin-4 Promotes Neurite Outgrowth by Chick Motor Neurons

It is important to investigate whether native laminin containing a β2 chain also inhibits motor neurite outgrowth since the relevant LRE sequence of the rat β2-chain is found within the α-helical assembly domain of the heterotrimeric laminin-4 molecule (Hunter et al., 1989*a*). We have previously shown that both isoforms, laminin-2 and -4, promote neurite outgrowth by chick sympathetic and sensory neurons (Brandenberger and Chiquet, 1995). We tested these two isoforms for their effect on neurite outgrowth by chick motoneurons. Both chick CG neurons (Pilar et al., 1980) and spinal cord motoneurons formed processes on laminin-2 as well as on laminin-4 substrates, although the latter sprouted more slowly on both isoforms (Fig. 2).

For CG neurons, outgrowth was quantified in terms of the percentage of sprouting neurons as a function of the laminin concentration used for coating (CG neurons; Fig. 3 *A*). In control experiments, the amount of laminin bound to the substrate was found to be proportional to the coating concentration and to be the same for both isoforms (see Materials and Methods). The percentage of sprouting CG neurons reached a maximum of ~70% at 1 μg/ml for both laminin-2 and -4. Higher concentrations resulted in only a slight further increase in the percentage of cells with neurites (Fig. 3 *A*). Since both isoforms exhibit a similar dose-response curve, growth on laminin-4 cannot be accounted for by an undetected contamination with laminin-2 (cf. Fig. 1). Moreover, the sprouting response of chick motoneurons to these two laminin variants was even more pronounced than their response to mouse laminin-1. After 8 h in culture, only ~50% CG neurons with neurites were observed on mouse laminin-1 at a saturating concentration (not shown), compared to the 70% on the the two chicken laminin variants.

Since laminin-4 and laminin-2 elicited the same response by motoneurons in terms of neurite growth, we asked whether this activity was mediated by a similar receptor. Therefore, we cultured CG neurons on the two isoforms in the presence of different anti-integrin antibodies. mAb JG22 against the chick integrin β1 subunit (Greve and Gottlieb, 1982) completely abolished neurite outgrowth on both isoforms. CG neurons express integrin α1, α3, α6, and α8 subunits on the cell surface (Weaver et al., 1995; Varnum-Finney et al., 1995). Whereas anti-integrin α3 and α6 (but not α1 or α8) antibodies reduced neurite growth by CG neurons on laminin-1, none of these antibodies were able to inhibit outgrowth on laminin-2 or -4 (data not shown). These findings agree with those found by Weaver et al. (1995) for human merosin (a mixture of laminin-2 and -4). Clearly, β1 integrin receptor(s) mediate neurite outgrowth by CG neurons in response to both laminin-4 and laminin-2; however, the identity of the α chain remains unknown.

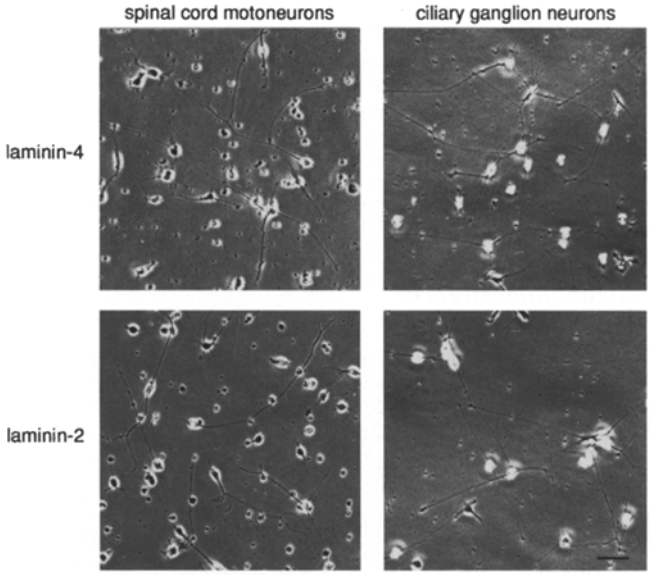

Figure 2. Laminin-4 (α2β2γ1) and laminin-2 (α2β1γ1) both promote neurite outgrowth by chick motoneurons. Chick spinal cord and CG motoneurons were plated on either laminin-4 or laminin-2 in medium containing serum and embryo extract. After 8 h (CG neurons) or 48 h (spinal cord neurons), cultures were fixed with formaldehyde and photographed. At the corresponding times, neurons on polylysine or BSA substrates, respectively, had formed neurites no longer than 2–3 cell diameters in average (not shown). Bar, 50 μm.

Chick spinal cord motoneurons were plated on laminin isoforms coated at 3 μg/ml. After 48 h in culture, ~50% of the surviving neurons had formed processes on both laminin-4 and laminin-2 (laminin-4, 51% ± 5%, n = 5, 662 neurons counted; laminin-2, 48% ± 4%, n = 3, 290 neurons). In addition, the distribution of neurite lengths was the same on both isoforms (Fig. 3 *B*). Thus, neurite outgrowth by both CG and spinal cord motoneurons on laminin-4 was indistinguishable from growth on laminin-2.

Motor Neurite Outgrowth on Laminin-2 and -4 in Chemically Defined Medium

In the experiments with motoneurons described above, we

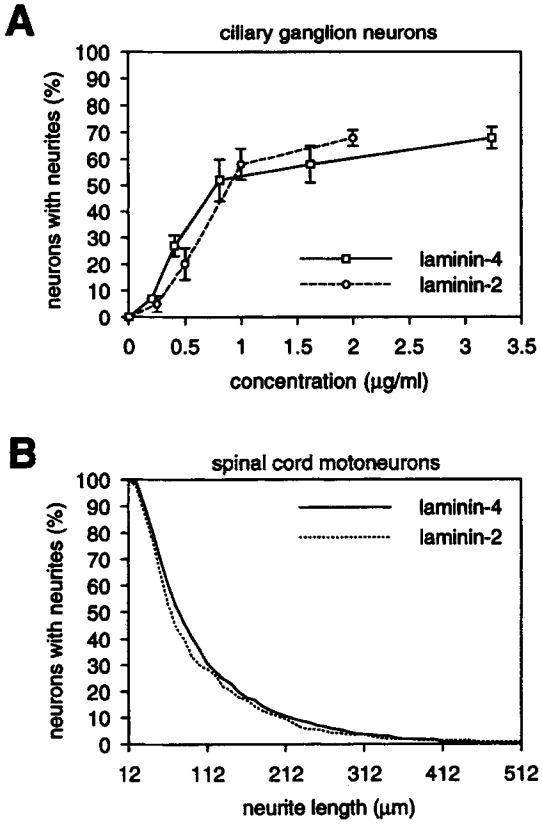

Figure 3. Quantification of neurite outgrowth promoted by chick laminin-4 and laminin-2. Chick motoneurons were cultured in medium containing serum and embryo extract. (*A*) Percentage of sprouting CG neurons on laminin-4 and laminin-2, respectively, coated at different concentrations. After 8 h, cultures were fixed and photographed. For both laminin isoforms, nearly maximal response was obtained at 1 μg/ml. Data are from one large experiment and three wells were coated per substrate. For each data point 4–5 photographed fields were measured and averaged; standard deviations are indicated. 372-684 neurons were counted per data point. A second independent experiment did not differ qualitatively. Moreover, no statistically significant difference in the percentage of sprouting neurons on saturating coating conditions of the two isoforms was found in six independent experiments. (*B*) Distribution of neurite lengths of spinal cord motoneurons cultured on laminin-4 or laminin-2 for 48 h. Data are given as the percentage of sprouting neurons (y-axis) with neurites longer than a given length (x-axis). Neurites of 602 (laminin-4) and 290 (laminin-2) neurons, respectively, were measured. No significant difference was found ($P < 0.05$). Independent experiments (3) gave the same result.

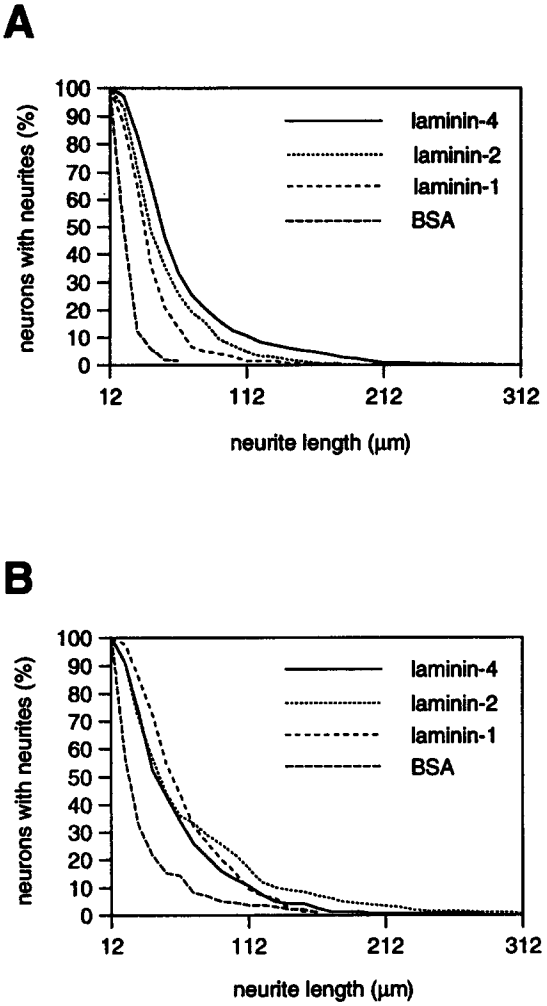

Figure 4. Neurite outgrowth by chick spinal cord motoneurons on laminin-2 and laminin-4 substrates in chemically defined medium. Cells were cultured in Ham's F-12 medium containing 1 mg/ml BSA and 10 ng/ml BDNF, fixed after 24 h (*A*) or 48 h (*B*), and the neurite length of individual neurons was measured (24 h: laminin-2: 555 neurons; laminin-4, 506 neurons; laminin-1, 124 neurons; BSA, 190 neurons; 48 h: laminin-2, 210 neurons; laminin-4, 165 neurons; laminin-1, 84 neurons, BSA, 114 neurons). Data are expressed as the percentage of sprouting neurons (y-axis) with neurites longer than a given length (x-axis). Data are from one representative experiment (three independent experiments were performed).

used complex medium containing serum and chick embryo extract. The aim was to reproduce the conditions for which the effect of recombinant rat laminin β2 chain fragments on chick motoneurons had been tested (Hunter et al., 1991; Porter et al., 1995). However, in such a medium, neurite outgrowth might be modulated or induced by unknown factors adsorbed to the substratum, especially in the case of spinal cord motoneurons which sprout slowly. Therefore, we cultured these neurons on laminin-1, -2, and -4, respectively, in defined F-12 medium containing only BSA and BDNF, and measured the length of their neurites at different times. Although neurites were somewhat shorter by average than those formed in complex medium, considerable growth was observed on all laminin isoforms but not on the control BSA substrate (Fig. 4). At 24 h, 40–50% of sprouting neurons had neurites longer than 50 μm on laminin-2 and -4, but <5% on BSA (Fig. 4 *A*). If anything, laminin-4 was slightly more active than the other isoforms at this time. After 48 h, neurite lengths were very similar on all three laminin isoforms and much higher than on the control substrate (Fig. 4 *B*). Moreover, the percentage of neurons with neurites did not differ between laminin-2 and laminin-4 (24 h: laminin-2, 43% ± 1%, n= 5, 655 neurons counted; laminin-4, 42% ± 3%, n = 11, 1890 neurons; laminin-1, 37% ± 3%, n = 4, 234 neurons; BSA, 14% ± 3%, n = 4, 722 neurons; 48 h: laminin-2, 47% ±

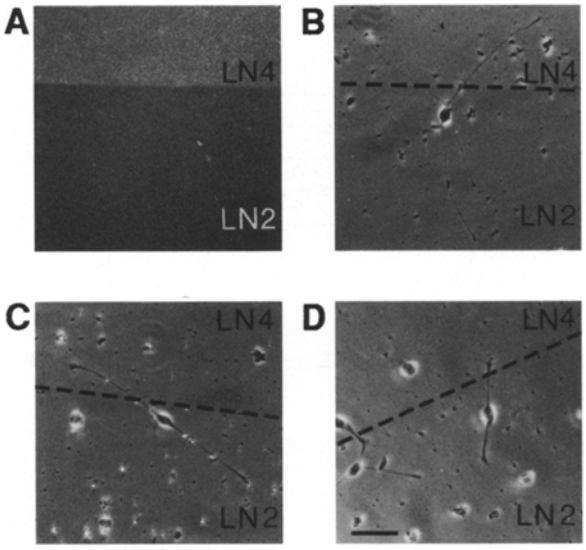

Figure 5. Motor neurites do not stop at borders between laminin-2 and laminin-4. A patterned substratum of laminin-2 and laminin-4 was prepared as described in Materials and Methods. The culture was fixed after 8 h. Borders between laminin isoforms were visualized by staining laminin-4 with mAb C4 against the β2 chain (*A*) and are indicated with dotted lines in *B–D*. Neurites readily grew from laminin-2 onto laminin-4 substrate (*B–D*). Bar, 50 μm.

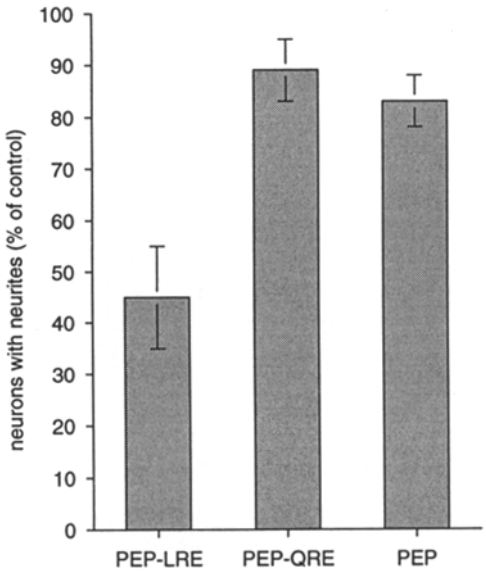

Figure 6. A LRE-containing fusion protein inhibits motor axon growth on laminin-1 (α1β1γ). Recombinant fusion proteins PEP-LRE and PEP-QRE were generated by fusing a small leader protein (PEP) to a rat β2 chain (s-laminin) derived peptide LRE-QVGDQYQ, to give PEP-LRE or to a control peptide, QRE-QVGDQYQ, to give PEP-QRE. Recombinant fusion protein was mixed with laminin-1 to give a final concentration of 20 μg/ml laminin-1 and 150 μg/ml fusion protein. CG neurons were cultured on the mixed substrate for 8 h. Only PEP-LRE inhibits neurite outgrowth whereas the leader protein alone (PEP) and the control peptide (PEP-QRE) have only very little effect. Data are from one experiment. Nine photographed fields were counted per substrate and averaged and the standard deviation is indicated. Between 672 and 1185 neurons were counted in total per substrate. In the controls (20 μg/ml laminin-1 alone), 47± 4% of the neurons had neurites. Independent experiments (5) gave similar results.

4%, n = 6, 365 neurons counted; laminin-4, 45% ± 4%, n = 5, 265 neurons; laminin-1, 39% ± 7%, n = 4, 211 neurons; BSA, 16% ± 2%, n = 4, 293 neurons).

In summary, we have shown that native chick laminin-4 which contains the β2 chain promotes rather than inhibits neurite outgrowth by chick motoneurons. The elicited growth response could not be distinguished from process formation on laminin-2 in which the β1 replaces the β2 subunit.

Neurites Do Not Stop at Borders between Laminin-2 and Laminin-4

Since regenerating axons stop at the original muscle endplates that are rich in laminin-4 (Sanes, 1995), we attempted to simulate this situation in vitro by generating a patterned substratum between laminin-4 and laminin-2, and asked whether such a border would affect neurite outgrowth by cultured CG neurons. The patterned substratum was created as described (Wehrle-Haller and Chiquet, 1993), neurons were plated and photographed after 8 h (Fig. 5). To visualize the border between the two isoforms, the cultures were stained with mAb C4 against the β2 chain to detect laminin-4. Laminin-2 which does not contain the β2 chain is not stained (Fig. 5 *A*). The neurites of CG neurons seemed to ignore the border between the two isoforms and grew readily from laminin-2 onto laminin-4 (Fig. 5, *B–D*). Hence, native laminin-4 does not act as a stop signal for growing chick motor axons in vitro.

LRE-Inhibition of Laminin-1 Mediated Neurite Outgrowth by Chick Motoneurons

In apparent contrast to the results presented above, a fragment of the rat laminin β2 chain containing the sequence LRE has been reported to selectively inhibit neurite outgrowth by motoneurons cultured on laminin-1, acting as a stop signal for these neurons (Porter et al., 1995). As a starting point for understanding this discrepancy, we generated a soluble recombinant fusion protein (PEP-LRE) consisting of a small 6-kD leader protein followed by the rat laminin β2 chain–derived decapeptide LRE-QVGDQYQ, and tested it for its inhibitory action on chick motor axon growth (Fig. 6). The decapeptide is not involved in defined structures after fusion to the carrier protein as judged by CD spectroscopy (not shown). 20 μg/ml mouse laminin-1 were mixed with 150 μg/ml fusion protein, the mixture was coated onto nitrocellulose-treated dishes, and neurons were plated. As controls, a fusion protein with the control peptide QREQVGDQYQ (PEP-QRE) or the leader protein alone (PEP) were used, respectively. The LRE containing fusion protein PEP-LRE inhibited neurite outgrowth by chick CG neurons (Fig. 6, PEP-LRE), whereas the leader protein alone or the control peptide only slightly affected neurite outgrowth on laminin-1 (Fig. 6, PEP, PEP-QRE). Lower concentrations of PEP-LRE still inhibited neurite outgrowth, but the effect was less pronounced (75 μg/ml: 62% of control; 38 μg/ml: 67% of control). The slight decrease in neurite out-

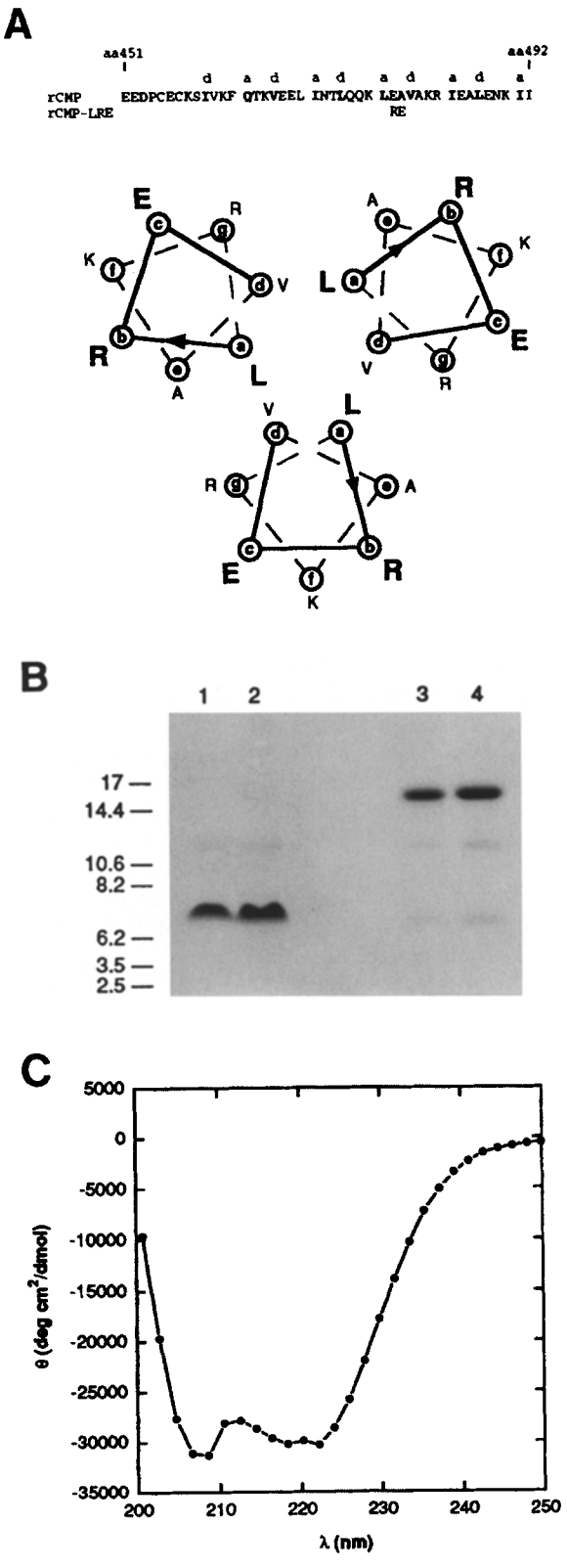

Figure 7. Characterization of the recombinant assembly domain of chicken cartilage matrix protein (rCMP). (*A*) Sequence of residues 451-492 of chicken CMP exons 7 and 8. The heptad repeat pattern with positions a and d occupied by nonpolar and hydrophobic amino acids is shown. Aspartate 479 and alanine 480 were mutated to arginine and glutamate, respectively, to give an LRE sequence. The position of the LRE in the triple coiled coil α helix is shown. (*B*) Recombinantly expressed CMP and CMP-LRE with the mutated sequence were purified from bacterial lysate as described in Materials and Methods. After affinity purification the oligohistidine leader was removed by thrombin cleavage. On tricine–SDS-PAGE under reducing (lanes *1* and *2*) and nonreducing (lanes *3* and *4*) conditions no difference between the wild-type rCMP and the mutated rCMP-LRE was observed. Both proteins were essentially purified as disulfide linked trimers and only small amounts of monomers and dimers were detected (lanes *3* and *4*). (*C*) Circular dichroism spectrum of the recombinant fragments rCMP-LRE at 23°C.

growth due to the control peptides was within the observed range (Porter et al., 1995). A similar, somewhat weaker inhibition of neurite outgrowth was observed when PEP-LRE was mixed to laminin-4 (not shown). Thus, we could confirm the inhibitory action of the LRE sequence on motor neurite growth in vitro.

LRE Inhibition Is Conformation-dependent

The discrepancy between the results obtained with native laminin-4 containing the β2 chain vs the recombinant fragments of the β2 chain in inhibiting neurite outgrowth might be explained by the altered structural context of the LRE sequence in recombinant, monomeric peptides compared to the native protein. The LRE sequence of the β2 chain lies within the triple-stranded coiled-coil region of the laminin molecule and is probably not exposed. To simulate this structural context, we expressed in *E. coli* the assembly domain of chicken cartilage matrix protein (CMP) consisting of exons 7 and 8. CMP is a disulfide-linked homotrimeric glycoprotein of 148 kD with heptad repeats in the carboxy-terminal extension domain consisting of amino acids 451-492 in chicken (Fig. 7 *A*, Hauser and Paulsson, 1994). Heptad repeats of the general structure $(abcdefg)_n$ with hydrophobic and nonpolar residues at positions a and d are the characteristic feature of proteins with a potential for coiled-coil formation (Cohen and Parry, 1990). This region has been shown to form a disulfide-linked, triple-stranded α-helical coiled coil (Haudenschild et al., 1995; Beck et al., 1996). As chicken CMP does not contain a LRE but instead an LEA sequence in this carboxy-terminal domain, we mutated amino acids 479 and 480 to arginine and glutamate, respectively (Fig. 7 *A*), to obtain rCMP-LRE with an LRE sequence. Upon purification of the recombinant fragments and subsequent removal of the oligohistidine leader by thrombin cleavage, both recombinant fragments rCMP and rCMP-LRE formed disulfide-linked trimers (Fig. 7 *B*). Only small amounts of monomer and dimer were detected. Sedimentation equilibrium measurements yielded molar masses of 18.5 kD for rCMP and 18.8 kD for rCMP-LRE. In addition, the fragment rCMP-LRE displayed a CD spectrum characteristic for an α-helical structure with minima at 208 and 220 nm (Fig. 7 *C*). The $[\Theta]222$ value of $-30{,}700$ deg cm^2 dmol^{-1} indicated a degree of α-helicity of 89% by assuming that a value of $-34{,}500$ deg cm^2 dmol^{-1} corresponds to a helix content of 100% for a 47–residue peptide and by correcting for the length dependence of $[\Theta]222$ (Chen et al., 1974). These data are consistent with a trimeric α-helical coiled-coil structure of the recombinant fragments.

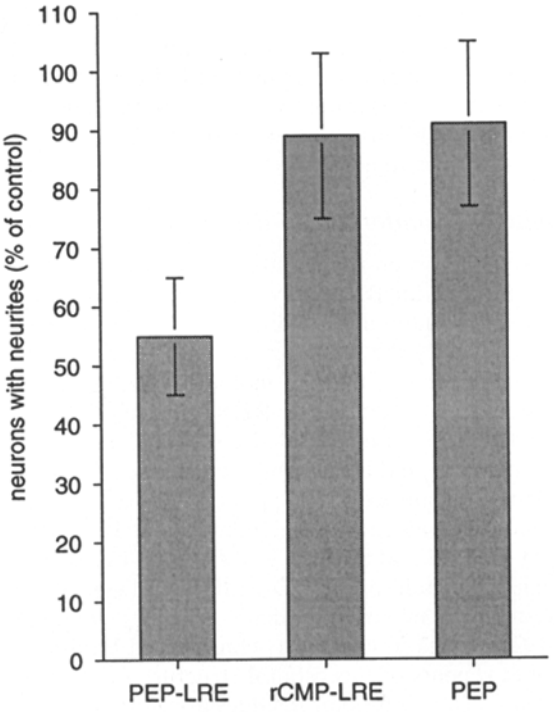

Figure 8. The inhibitory action of LRE is conformation-dependent. The triple coiled-coil fragment rCMP-LRE was tested for inhibition of motor axon growth on laminin-1. CG neurons were grown for 8 h on mixtures of 20 μg/ml laminin-1 and 180 μg/ml rCMP-LRE or 150 μg/ml PEP-LRE or PEP to obtain equimolar amounts of the three peptides (20 μM). No significant reduction of the percentage of neurite outgrowth on laminin-1 mixed with rCMP-LRE was found as compared to the control protein PEP. PEP-LRE which has no defined tertiary structure significantly inhibited process formation. Data shown are from one experiment. 7–8 fields per substrate were photographed, measured, and averaged; standard deviations are indicated. In total 364-585 neurons were counted per substrate. In the controls (20 μg/ml laminin-1 alone), 49 ± 6% of the neurons had neurites. Results between independent experiments (4) did not differ qualitatively.

The mutated CMP assembly domain, rCMP-LRE, was tested for its effect on CG neurons (Fig. 8). In contrast to the pronounced effect of PEP-LRE, rCMP-LRE had no effect on neurite outgrowth by CG neurons on laminin-1 (Fig. 8, CMP-LRE). The observed slight reduction of neurite outgrowth was not significantly different from the reduction obtained with the control protein PEP (Fig. 8, PEP), and, in a parallel experiment, this reduction was also observed with the wild-type CMP assembly domain (not shown). Thus, the LRE sequence does not inhibit neurite outgrowth when embedded in a triple-stranded α-helical coiled-coil structure.

Discussion

Because we are interested in the functional roles of distinct laminin isoforms in neural development, we isolated native laminin from chick tissues and separated variants by sequential immunoaffinity chromatography using subunit-specific antibodies (Brubacher et al., 1991; Brandenberger and Chiquet, 1995). We used the purified isoforms as substrates for different types of cultured chick neurons and quantified their neurite growth. As published previously, our methods allow us to distinguish between the activities of different laminin variants. For example, we showed that laminin isoforms with an α2 chain (merosin) are distinctly more active in promoting neurite growth by sympathetic neurons than those with a 200-kD αx chain, and about as active as laminin with an α1 subunit (Brandenberger and Chiquet, 1995). Here, we compared the activities of two native chick laminin isoforms which share α and γ but differ in their β subunits: laminin-2 (α2β1γ1) and laminin-4 (α2β2γ1). Essentially, we show that the rate of neurite outgrowth on these two laminins is identical for all neurons tested, in particular also for two types of embryonic chick motoneurons. Moreover, growing neurites do not recognize substrate borders between laminin-2 and -4: their growth cones continue to move at the same speed and direction.

For chick motoneurons, both laminin-2 and -4 seemed to be somewhat more active than laminin-1. Weaver et al. (1995) found similar differences in activity between mouse laminin-1 and human merosin which is a mixture of both laminin-2 and laminin-4 (Engvall et al., 1990; Brown et al., 1994). Neither the location of the neurite-promoting site within these two laminin isoforms, nor the neuronal receptors involved are known yet. Neurite outgrowth on both chick laminin-2 and laminin-4 seems to depend on neuronal integrin(s) of the β1 family, as has been shown before for merosin (Weaver et al., 1995). In accordance with published data (Weaver et al., 1995), neurite outgrowth on chick laminin-2 and -4 was not blocked by antibodies against the α subunits of known laminin-1 receptors, i.e., α1, α3, and α6. Which other integrin receptor of the β1 class is involved remains to be determined.

What is the importance of the result that native laminin-4 is as active as laminin-2 in inducing process formation by embryonic motoneurons? Laminin-2 is the common isoform in extrasynaptic muscle basement membranes, whereas laminin-4 is highly enriched at the neuromuscular junction (Engvall et al., 1990; Sanes et al., 1990). Laminin-4 but not laminin-2 has a β2 subunit (s-laminin). A recombinant COOH-terminal fragment of the rat β2 chain is adhesive for motoneurons, and their neurites stop growing when they reach a substrate containing this fragment (Porter et al., 1995). The peptide LRE contained within this part of the β2 chain sequence was shown to be required for inhibitory activity (Hunter et al., 1991). However, the apparent discrepancy between the data obtained with recombinant β2 chain fragments or peptides and our results with native, assembled laminin-4 needs to be explained.

It is well known that the secondary and tertiary conformation of oligopeptide sequences in proteins influences their interactions with ligands. For example, many of the RGD sequences found in ECM proteins are "cryptic," i.e., they are only recognized by integrin receptors after denaturation of the protein. In the rat laminin β2 chain, the LRE sequence in question is located in the α-helical triple-stranded coiled-coil region of the long arm of native laminin, i.e., in the assembly domain of the three subunits. Potential of an amino acid sequence to form an α-helical coiled coil is provided by the so-called heptad repeat $(abcdefg)_n$ where residues "a" and "d" are predominantly hy-

drophobic and face neighboring α-helices and hence are buried, whereas the other positions are frequently occupied by polar amino acids pointing to the outside (Cohen and Parry, 1990; Fig. 7 *A*). We evaluated the secondary structure of the rat laminin β2 chain amino acid sequence by applying the COILS2 algorithm (see Materials and Methods). The program compares any amino acid sequence with sequences of known coiled-coil sequences in a data base. In the case of the rat laminin β2 chain, this program predicts three stretches of α helix from residue 1265-1301, 1475-1529, and 1581-1786 in the long arm region, with a probability of 0.99. The COILS2 program assigns the leucine residue of the LRE sequence in question to a heptad "a" position. Therefore, in native laminin-4, this leucine is likely to be buried in the interior of the triple-stranded coiled coil. Since mutation of LRE to QRE abolishes all effects on motoneurons attributed to rat laminin β2 chain fragments and peptides (Hunter et al., 1989*a*, 1991; Porter et al., 1995), exposure of the leucine seems to be necessary for activity. This is probably the case in single chain fragments of β2 subunits, which are likely to have a random conformation because isolated α-helices are known to be unstable in aqueous solution unless they have a special sequence (Hunter et al., 1992; Pikkarainen et al., 1992). However, since the concept of coiled-coil formation requires the assembly of two or more α-helices, we propose that the corresponding site in native laminin-4 is hidden and inactive. To test this hypothesis directly, we generated LRE peptides in different conformations. With a randomly coiled LRE peptide, we were able to reproduce the published effects on chick embryonic motoneurons (Hunter et al., 1991; Porter et al., 1995). In contrast, a trimeric recombinant peptide with the LRE sequence incorporated into an α-helical triple-stranded coiled-coil conformation was inactive.

It should be noted that the LRE tripeptide occurs once every 2800 residues in random protein sequences (Hunter et al., 1989*a*), and it is even much more frequent in α-helical coiled-coil domains (once per 360 residues) as calculated from the frequency of these residues in known triple-stranded coiled coils (Woolfson and Alber, 1995). The accumulation of the three-residue sequence LRE in coiled-coil sequences is not unusual. In fact, L and E are the most predominant residues found in seven-residue repeats, and the three residues L, R, and E make up 30% of the amino acids in triple-stranded coiled coils (Woolfson and Alber, 1995). L is most frequent in heptad positions "a" and "d," whereas amino acids R and E are most often found in exposed positions where they play an important role in interchain ionic interactions. Charged residues in positions "e" and "g" are also known to be important determinants of stoichiometry and orientation of coiled-coil domains (Cohen and Parry, 1990). Expectedly, many intracellular coiled-coil proteins like cytokeratins contain LRE sequences (Hunter et al., 1989*b*). In the oligomeric ECM protein tenascin-c, an LRE is found in the triple-stranded coiled-coil assembly domain (Spring et al., 1989). One or more LRE sequences are also found in the laminin α1, α2, and γ1 subunits in several animal species, hence laminin isoforms 1 and 2, which clearly do not cause motor axons to stop growing contain LRE sequences. Therefore, even in the case of laminins it is obvious that most if not all of these LRE sequences must be cryptic. It is unlikely that this tripeptide can act as a signal that mediates specific reinnervation when it is present in a coiled-coil domain. Moreover, it has recently been reported that the LRE sequence at the COOH terminus of the laminin β2 chain, although conserved in the mouse (Brandenberger, R., and R.A. Kammerer, unpublished observation), is not present in the human protein (Wewer et al., 1994), again casting doubt on its significance in the native protein.

This by no means implies that the laminin β2 chain has no distinct function necessary for life. Recently, a gene knockout of this subunit has been generated (Noakes et al., 1995*a*, *b*). Mice lacking the laminin β2 chain die shortly after birth apparently from kidney failure, which is not surprising in view of the high glomerular expression in wild-type animals. Moreover, in laminin β2-deficient mice the neuromuscular junctions are indeed abnormal in structure. However, synapses do develop at the expected sites on the muscle fiber surface, and motor axons do not produce abnormal terminal axon sprouts as, e.g., in mice overexpressing GAP-43 (Aigner et al., 1995). These as well as our studies argue against the hypothesis that the laminin β2 chain directs the growth of motor axons to the synaptic site during development and regeneration. Hence, the putative function of this laminin subunit should be reevaluated. Since it is found in the basement membranes of specialized cell–cell contacts such as the myotendinous and the neuromuscular junction and in the glomerular basement membrane, the laminin β2 chain might be essential for the mechanical stability and maintenance of these structures. To find out, we are studying the specific interactions of native laminin containing a β2 subunit with other ECM proteins as well as with cells.

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